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Bromelain Administration Ameliorates Neurobehavioural Deficits Mediated by Cadmium Neurotoxicity via Oxido-Nitrosative Stress, Cholinergic and Neuroinflammatory Modulations in Male Wistar Rats

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ABSTRACT

Cadmium (Cd) is a toxic metal widely present in the environment. Certain plant-based natural or active compounds are gaining attention for their neuroprotective effects against chemically induced toxicity. However, there is no report on the impact of bromelain on Cd-induced neurotoxicity, hence the rationale behind this study. Twenty-four adult male rats were divided into four groups: control (normal saline, 5mL/kg), Cd (orally, 5mg/kg), bromelain (50mg/kg), and Cd+bromelain. Rats received bromelain or normal saline for 14 days. Cd was administered concurrently with bromelain and normal saline in the last 7 days. Neurobehavioural responses to locomotion, cognition, anxiety, and depression were assessed. Plasma was assayed for the levels of superoxide dismutase (SOD) and malondialdehyde (MDA), while the prefrontal cortex (PFC) was processed for the concentration of Cd and levels of SOD, MDA, nitric oxide (NO), acetylcholinesterase (AChE), interleukin-6 (IL-6), and tumour necrotic factor-α (TNF-α). Data were analysed using one-way ANOVA (Tukey's post hoc), with the level of significance set at $p<0.05$. Exposure to Cd caused a significant behavioural deficit, as indicated by a decline in motor coordination, memory index, and enhanced depressive and anxiety-like behaviours. Biochemically, Cd concentration was significantly increased in the PFC of Cd group compared to the control. There was a reduction in the level of SOD and increased levels of MDA, NO, IL-6, AChE, and TNF-α following Cd exposure. Bromelain significantly improved the behavioural outcomes and reversed some of the biochemical markers altered by Cd toxicity. Bromelain reduced neurobehavioural impairment, cholinergic alteration, and oxido-inflammatory deficits induced by Cd exposure.

Keywords

Bromelain, Cadmium, Oxido-nitrosative stress, Neuroinflammation, Neurotoxicity

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INTRODUCTION

Cadmium (Cd) is a severely toxic metal widely present in the environment and noxious to human health as it is known to bioaccumulate in the human body due to its long biological half-life (Genchi *et al*., 2020). Oxidative stress, cholinergic alteration, and neuroinflammation are the primary mechanisms underlying heavy metal-induced neurotoxicity (Zhao *et al*., 2020). The beneficial effects of certain natural or active compounds from plants against chemically induced toxicity are gaining more attention. Some phytochemicals from plants are known to have neuroprotective properties. However, there is no report on the effect of bromelain on Cd-induced neurotoxicity.

Humans typically get exposed to Cd by inhalation and ingestion of Cd-contaminated air, food, and water (Zhang

and Reynolds, 2019). It accumulates in the kidneys, liver, central and peripheral nervous systems, and other tissues, where it exerts toxic and carcinogenic effects (Wang and Du, 2013). In animals, Cd induces neurotoxicity, including oxidative stress, changes in the typical structure and neurochemistry of the brain, apoptosis, inflammation, altered locomotion, enhanced anxiety and depression, memory impairment, and altered brain enzyme activities (Hossein-Khannazer *et al*., 2020; Zhang *et al*., 2020). Moreover, several clinical investigations have linked Cd poisoning as a possible aetiological factor for neurodegenerative diseases, including Parkinson's, Alzheimer's and Huntington's diseases (Chong *et al*., 2017).

Impaired behaviours, including cognitive, motor-, and anxiety-related outcomes following Cd exposure, have been argued to involve the cholinergic system via alteration of acetylcholinesterase (AChE) activity (Goncalves *et al.,* 2013; Gupta *et al.,* 2017). Cd exposure also elicits inflammation in the brain, as determined by the overexpression of NF-κB and IFN-γ (Almeer *et al*., 2019). This inflammatory response to Cd was also reported in mouse brain cortical neurons by the enhancement of tumour necrotic factor-α (TNF-α) and interleukin 1β. Accumulation of Cd in the brain enhances the production of reactive oxygen species (ROS) (El-kott *et al*., 2020), as well as reactive nitrogen species (RNS) comprising nitric oxide (NO), which are released from inflamed neuronal cells and neutrophils, leading to oxido-nitrosative stress (Kandikattu, 2018).

Naturally, antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione scavenge ROS to nullify their oxidative effects (Jeeva *et al*., 2015). However, Cd induces oxidative stress via inhibition of these antioxidant enzymes, lipid membrane peroxidation, and protein oxidation. Intraperitoneal administration of Cd to rats reportedly led to a decrease in the activity of SOD and an increase in the brain concentration of lipid peroxide (Renugadevi and Prabu, 2010; Oboh *et al*., 2019).

Environmental pollution associated with nutritional deficiency is an important factor that contributes to neurological disorders due to metal poisoning; therefore, the prevention and management of such diseases with a healthy diet have now become a major concern for researchers. A good diet includes the ingestion of foods that contain micronutrients with antioxidant, anti-inflammatory, and chelating properties. Several studies have demonstrated that Cd brain intoxication can be reverted by compounds such as curcumin, N-acetylcysteine, cysteine, quercetin, melatonin, vitamin E, resveratrol, omega-3, and lots more (Almeer *et al*., 2019; Alnahdi and Sharaf, 2019). Moreover, the administration of a diet rich in plant-based foods and drinks has been shown to alleviate Cd-induced toxicity in different animal models (Akinyemi and Adeniyi, 2018; Ali *et al*., 2019).

Bromelain is a cysteine proteolytic enzyme derived mainly from pineapple stems. It has been scientifically proven for its anti-inflammatory, anti-carcinogenic, anti-nociceptive, and antioxidant properties (Habashi *et al*., 2016; Saptarini *et al*., 2019). Its neuroprotective effects have been widely reported, such as in chronic constriction injury-induced neuropathic pain in Wistar rats (Bakare and Owoyele, 2020), a 6-OHDA-induced *in vitro* model of Parkinson's disease (Ferrah *et al*., 2021), aluminium and D-galactose-induced Alzheimer's disease in rats and via modulation of the TXNIP pathway (Kumar *et al*., 2022; Eraky *et al*., 2023). Bromelain, the cysteine protease from pineapple, has broad specificity. It offers a wide range of therapeutic efficacies and due to its efficiency after oral administration, safety, and lack of undesired side effects, bromelain is being increasingly accepted as a phytotherapeutic compound (Maurer, 2001). Cysteine-containing compounds have been documented to bind, chelate, and excrete metals from the human body (Al-Otaibi *et al*., 2015). Since bromelain also contains cysteine and sulfhydryl, we hypothesise that it could potentially combat Cd-induced neurotoxicity. On this basis, the present study explored the potential ameliorative effects of bromelain administration on behavioural deficits, cholinergic alteration, neuro-inflammation, and oxidative stress associated with Cd neurotoxicity.

MATERIALS AND METHODS

Experimental Animals

Twenty-four adult male rats ranging in weight between 117 and 125 g were obtained from the central animal house, University of Ibadan. The rats were kept in the animal facility of the Faculty of Basic Medical Sciences, Adeleke University, Ede, in polypropylene plastic cages (42×30 x 27 cm) with wood shavings as bedding at a room temperature of 27–30 °C with a 12 h light/dark cycle. Rats were allowed to have access to standard rodent pellet food and water ad libitum throughout the experimental period. Rats were acclimatised for at least one week before the experiments. The experimental protocol for this study was approved by the Adeleke University Animal Care and Use ethical review committee (Ethical approval number: AUERC/FBMS/20) in strict compliance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (National Research Council, 2011).

Drug/Chemical Preparations and Dosages

The doses of bromelain (KAN Phytochemicals, Rai, India) (50 mg/kg) (Bakare and Owoyele, 2020; Bakare and Owoyele, 2021) and Cd chloride (Sigma Aldrich, Missouri, USA) (5 mg/kg) (Shagirtha *et al*., 2011; Oboh *et al*., 2019; Vijaya *et al*., 2020) were selected according to findings from preliminary and previous reports. Saline (0.9%) was used to constitute bromelain and Cd prior to oral (p.o.) gavage administration and was administered in a volume of 5 mL/kg of individual animal weight.

Experimental Design

Twenty-four male rats were randomly divided into four groups of six rats each. Control (normal saline, orally, 5 mL/kg), Cd (orally, 5 mg/kg), bromelain (50 mg/kg, oral), and Cd+bromelain. Rats received bromelain or normal saline for 14 days. Then, Cd was administered concurrently with bromelain and normal saline in the last seven days. In the combined group, bromelain was administered 30 min

before Cd. Neurobehavioural responses to motor activities (horizontal bar and open field tests), cognitive function (novel object recognition test), anxiety (elevated plus maze test), and depression (forced swimming test) were assessed in the last two days. On day 15, rats were anaesthetized using a ketamine/xylazine cocktail (100/10 mg/kg). Blood (plasma for biochemical assay) was taken via cardiac puncture. The brain was removed, and the prefrontal cortex (PFC) was isolated and processed for biochemical analysis of Cd, malondialdehyde (MDA), superoxide dismutase (SOD), nitric oxide (NO), and acetylcholinesterase (AChE) spectrophotometrically. Prefrontal cortical levels of interleukin 6 (IL-6) and tumour necrotic factor-α (TNF-α) were determined using an enzyme-linked immunosorbent assay (ELISA).

Behavioural Assessment

Behavioural tests were carried out in the last two days of administration of saline, bromelain, and/or Cd on days 13 and 14. The open field test for motor assessment and the novel object recognition test for assessment of cognitive function were done on day 13, while the elevated plus maze test for assessment of anxiety-like behaviour and the forced swimming test for assessment of depressive-like behaviour were performed on day 14. All behavioural tests were carried out between 8:00 a.m. and 2:00 p.m. by trained observers who were blind to the treatment groups.

Open Field Test: As described by Seibenhener and Wooten (2015), each rat was placed in the centre of the open field maze and recorded using a video camera. Ambulatory (horizontal exploratory) activity, rearing (vertical exploratory) behaviour, centre exploration, and freezing behaviours were assessed for 6 min (Fedotova *et al*., 2017). Centre exploratory activity was used to assess anxiety-like propensity. Lower times spent in the central region of the open field imply anxiogenic action (Kraeuter *et al*., 2019).

Novel Object Recognition Test: This test assesses non-spatial short-term or working memory in animals as initially described by Bevins and Besheer (2006), but with slight modification by Bayo-Olugbami *et al*. (2020). A 45 × 50 cm opaque box was used. Rats were allowed to explore two identical objects placed 5 cm apart from each other and from the walls of the box for 10 min (Trial 1; T1) (training section). An inter-trial time (resting phase) of about 30 min was observed, after which the rat was returned to the testing field for the second trial (T2). The time allowed for T2 was also 10 min, during which one of the old objects was replaced with a novel object (the test session). Exploration was scored when the nose or vibrissae of the rat was about 2 cm from the object, while sitting on the object was excluded. The box arena and objects used were thoroughly wiped with 70% ethanol after each test before introducing the next rat in order to reduce olfactory cues (Bevins and Besheer, 2006). The time spent exploring the old object in T1 and the new object in T2 was used to estimate the memory index, calculated as [time spent exploring the new object/total time spent exploring both objects] × 100.

Elevated Plus Maze Test (EPM): The EPM is an ethological measure used to assess anxiety-like behaviour (Mittal *et al*., 2016). The apparatus used was a wooden maze in the configuration of a plus when viewed from above, as described by Handley and Mithani (1984), but slightly modified. It consists of two open arms perpendicular to two wall-enclosed arms and a centre platform. A rat was placed at the intersection of the four arms, facing one of the open arms. The theory underlying the EPM is based on the known tendency of rodents to favour enclosed, dark spaces, to have an unconditioned fear of open spaces and heights, and to have an intense, innate desire to explore novel environments. Behaviour is frequently recorded during a 5-minute test period because animals exhibit the most robust avoidance responses within that period. The duration of time spent in the closed arm of the EPM was recorded. A high closed arm duration or entry or a low open arm duration or entry is indicative of anxiety, and vice versa. The animal is deemed to be in an area when the centre of its body mass is in that area (Mittal *et al*., 2016).

Forced Swimming Test: FST was assessed by a slight modification of the method described initially by Porsolt *et al*. (1977). Briefly, a transparent cylinder (60 × 20 cm) was filled with water to a depth of 30 cm. For training, each rat was placed inside the cylinder for 10 min. Then, the rats were dried and placed back in their cages. Twenty-four hours later, the rats were tested and recorded for 6 min using a video camera. The two parameters assessed were (1) immobility time (floating in the water with only movements necessary to keep the head above water) and (2) swimming time (active swimming movements around the glass cylinder). Higher immobility time indicates depressive-like behaviour (Yankelevitch-Yahav *et al*., 2015).

Animal Sacrifice, Sample Collection, and Preparation of Tissues for Biochemical Assays

After behavioural evaluation, rats were anaesthetized with a ketamine (100 mg/kg)/xylazine (10 mg/kg) cocktail. Blood was collected via cardiac puncture into heparinized tubes and centrifuged at 5,000 rpm for 15 min in order to obtain plasma for biochemical assays of SOD and MDA. Afterward, transcardial perfusion was done using 50 mL of 0.1 M PBS (pH 7.4). Each rat was decapitated, the whole brain was removed, and the PFC was dissected and rinsed in 0.25 M sucrose. PFC was homogenised over ice in 0.1 M cold sodium phosphate buffer (pH 7.4). The homogenate was centrifuged at 4 ℃ for 10 min at 10,000 rpm. The supernatant obtained was aliquoted to determine oxido-nitrosative stress, inflammatory parameters, and acetylcholinesterase activity.

Determination of Cd Concentration in PFC

The PFC was digested according to Babalola *et al*. (2009). Thereafter, the Cd ion concentration of the digested tissue was measured using an atomic absorption spectrophotometer. The analytical blank was run the same way as the samples, and the standard solution for the calibration curve was prepared in the same matrix. The Cd concentration in brain tissue was expressed as ng/g dry weight.

Determination of Oxidative and Nitrergic Stress Markers

Superoxide Dismutase (SOD): The activity was determined according to the method of Sun *et al.* (1988). The principle of the method is based on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine-xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the lysate after a 1.0-mL ethanol-chloroform mixture (5/3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate.

Malondialdehyde (MDA): The level of MDA was determined indirectly as thiobarbituric acid reactive substances (TBARS) according to the thiobarbituric acid reaction of Mihara and Uchiyama (1978). Briefly, 3 mL of 1% H_3PO_4 and 1 mL of 0.6% TBA aqueous solution were added to 0.5 mL of 10% homogenate of the tissue sample. It was stirred, and the mixture was heated in a boiling water bath for 45 min and allowed to cool. Then, 4 mL of n-butanol was added and shaken together, and the butanol layer was separated by centrifugation. The optical density was read at 535 and 520 nm.

Nitric Oxide (NO): Nitric oxide was assayed according to a previous method (Tracey *et al*., 1995) using the Griess reagent system with a few modifications (Sun *et al*., 2003). 0.1% w/v NED solution (naphthyl ethylene diamine dihydrochloride) was used. The reaction mixture containing prefrontal cortical homogenate (50 µL supernatant) and phosphate-buffered saline (50 µL) was incubated at 25 °C for 15 min. Then 50 µL of sulphanilamide solution (1% sulphanilamide in 5% phosphoric acid) was added and allowed to sit for 5 min. The absorbance was measured at a wavelength of 540 nm against the corresponding blank solutions. Sodium nitrite was used as a standard sample.

Determination of Cholinergic Marker

Estimation of Acetyl-Cholinesterase Activity: The procedure described by Ellman *et al*. (1961) was used to estimate AChE activity in the PFC. Briefly, a 50 mL aliquot of brain supernatant was diluted with 50 mL of phosphate buffer (0.1 M, pH 7.4), followed by the addition of 50 mL of 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB, 0.0001 M) in a 96-well plate. The initial absorbance was first measured after 5 min of incubation with DTNB. Thereafter, 50 mL of acetylthiocholine iodide (0.028 M) was added to the mixture for 3 min, and the absorbance was again measured at 405 nm in a microplate reader (Micro READ 1000, Belgium).

Determination of Neuro-Inflammatory Markers

The concentration of IL-6 and TNF-α in the PFC was determined using enzyme-linked immunosorbent assay kits (Nanjing Mornmed Medical, Nanjing City, Jiangsu Province, China) following the manufacturer's guidelines. All the measurements were done at room temperature using a microplate reader with a 450 nm filter (Micro READ 1000, Belgium). The concentrations of IL-6 and TNF-α in the PFC were extrapolated from the standard curve and expressed as pg/mg protein.

Statistical Analysis

GraphPad Prism version 8.0 was used for all statistical analyses. All data were expressed as mean±SEM. Data were analysed using one-way ANOVA and Tukey's post hoc for multiple comparisons. A P value < 0.05 was considered to be statistically significant.

RESULTS

Effects of Bromelain on Cd Concentration in PFC in Cd-Induced Neurotoxicity in Rats

Exposure to Cd caused a significant increase in the deposition of Cd in the cortex compared with control ($p<0.05$). A similar result was observed in the group administered Cd+bromelain compared with the control group. However, the bromelain-only ($p < 0.05$) and Cd+bromelain ($p < 0.05$) groups showed a significant reduction in Cd deposit compared with rats exposed to Cd without intervention. Cd accumulation was also increased in rats administered Cd+bromelain ($p<0.05$) compared with the bromelain-only group (Fig. 1).

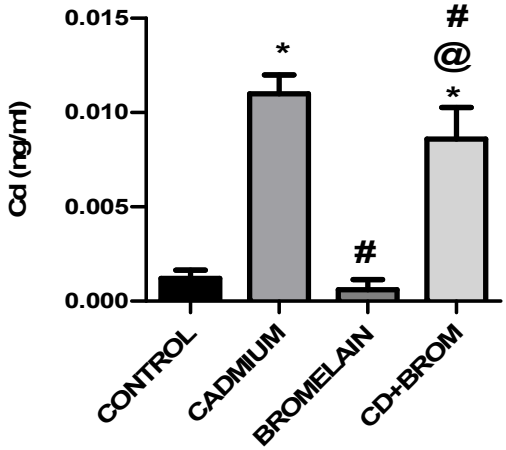

Fig 1: Effects of bromelain on the concentration of Cd in the PFC of cadmium exposed rats. Values are expressed as mean ± SEM (n=5). *P<0.05 vs control; #P<0.05 vs Cd; @P<0.05 vs bromelain

Effects of Bromelain on Behavioural Assessment in Cd-induced Neurotoxicity in Rats

Cd exposure reportedly affects behaviour. As such, we investigated the impacts of Cd on motor, cognitive, anxiety-, and depressive-like behaviour using an appropriate toolbox and also determined the effects of bromelain on such deficits.

Effects of Bromelain on Motor or Exploratory Activities in OFT in Cd-Induced Neurotoxicity in Rats: In Figure 2A, ambulatory or exploratory activity, as depicted by the total number of lines crossed in the open field test, was markedly reduced in the Cd-exposed rat ($p<0.05$) compared with control rats. In contrast, rats that received bro-

melain only (p<0.05) and Cd+bromelain (P<0.05) had an increased number of lines crossed when compared with the Cd group, depicting an improvement in exploratory function.

Rearing frequency: As shown in Figure 2B, rearing frequency, an index of vertical exploratory activity, was significantly lower in the Cd group than the control (p<0.05). Conversely, this was increased in the bromelain (p<0.05) and Cd+bromelain (p<0.05) groups compared with the untreated Cd group.

Frequency of Centre Exploration: The frequency of centre exploration in the open field maze is used to estimate anxiety-like behaviour in rodents. In Figure 2C, the frequency of centre visits in Cd-exposed rats was markedly low compared with the control (p<0.05). Bromelain only (P<0.05) and Cd+bromelain (p<0.05) had a significant increase in centre frequency compared with the Cd group. Cd+bromelain -treated rats (p<0.05) also showed a marked reduction in the number of times rats visited the centre of the maze compared with the bromelain-only group.

Freezing frequency: Freezing frequency, which was the number of times rats stayed at a point immovable, was significantly increased in the Cd group (p<0.05) compared with the control. Only bromelain-treated rats (p<0.05) showed a marked decrease in freezing frequency compared with the Cd group (Fig. 2D).

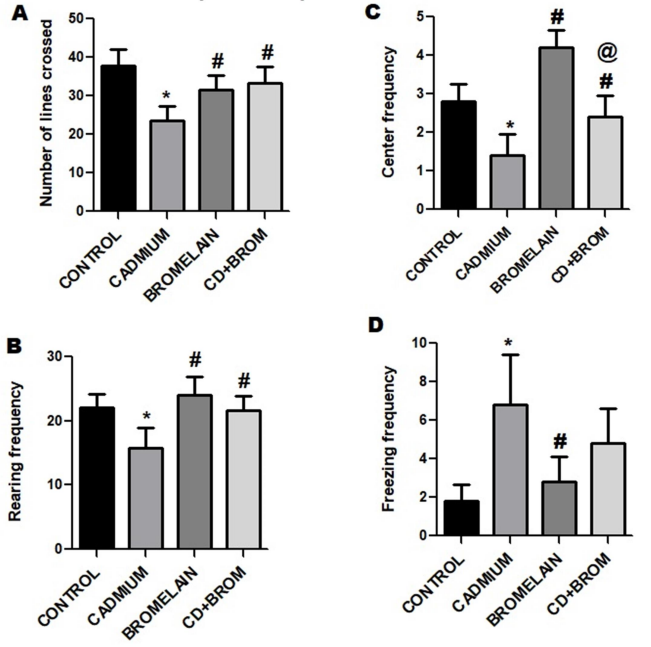

Fig 2: Effects of bromelain during an open field test in Cd-induced neurotoxicity in rats. A - the number of lines crossed; B - rearing frequency; C - frequency of centre exploration; D - freezing frequency. Values are expressed as mean ± SEM (n=5). *P<0.05 vs control, #P<0.05 vs Cd (cadmium), @P<0.05 vs bromelain.

Effects of Bromelain on Cognitive Behaviour in NOR in Cd-Induced Neurotoxicity in Rats

In Figure 3, the memory index, which measures the percentage of preference for new object exploration, was observed to be significantly reduced in Cd-exposed rats (p<0.05) compared with the control. However, this was reversed in rats that received bromelain only and Cd+BROM (p<0.05) when compared with the Cd group that received no intervention, depicting an improvement in memory function.

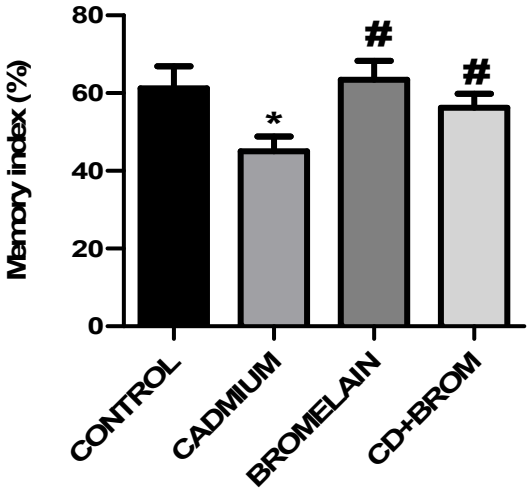

Fig 3: Effects of bromelain on memory index during novel object recognition test in Cd-induced neurotoxicity in rats. Values are expressed as mean ± SEM (n=5). *P<0.05 vs control, #P<0.05 vs Cd (cadmium).

Effects of Bromelain on Mood (Anxiety- and Depression-Like) Behaviours in Cd-Induced Neurotoxicity in Rats

In Figure 4A, anxiety-like behaviour was depicted by the increased duration of time spent in the closed arm of EPM, which was significantly higher in Cd-exposed rats (p<0.05) compared with the control. Conversely, bromelain (p<0.05) and Cd+bromelain (p<0.05) had a marked decline in closed-arm duration when compared with the Cd group. In addition, the duration was higher in the Cd+bromelain (p<0.05)-treated rats compared with the bromelain-only group.

In Figure 4B, the immobility time was significantly increased in the Cd group compared with the control (p<0.05). Intervention with bromelain only and Cd+bromelain (p<0.05) markedly reduced the immobility time compared with the Cd-only group, depicting a reduction in depressive-like propensity. Also, the Cd+bromelain group showed a significant increase in immobility time compared with the bromelain-only group (p<0.05).

Effects of Bromelain on Markers of Oxidative Stress, Cholinergic Transmission, and Neuro-Inflammation in Plasma or PFC in Cd-Exposed Rats

The levels of some biochemical markers of oxidative stress (SOD, MDA, and NO), cholinergic transmission (AChE), and inflammation (IL-6 and TNF-α) in the plasma or PFC cortex of Cd-exposed rats were determined.

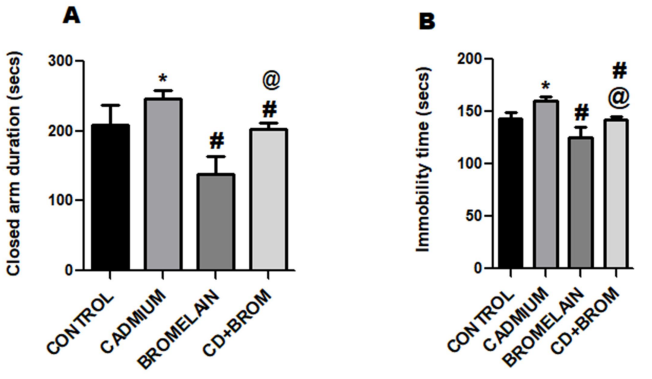

Fig 4: Effects of bromelain on Cd-induced neurotoxicity in rats. A - closed arm duration (anxiety-like behavior; B - immobility time (depressive-like behaviour). Values are expressed as mean ± SEM (n=5). *P<0.05 vs control, #P<0.05 vs Cd (cadmium), @P<0.05 vs bromelain.

Effects of Bromelain on the Plasma Level of Oxidative Stress Markers in Cd-Induced Neurotoxicity in Rats

SOD: In Figure 5A, the plasma level of SOD was low (p<0.05) in Cd-exposed rats compared with the control. However, bromelain only (p<0.01) and Cd+bromelain (p<0.05) showed a significant increase in the level of SOD when compared with the Cd group.

MDA: In Figure 5B, the level of MDA, a marker of lipid peroxidation and oxidative stress, was observed to be markedly increased in the Cd-exposed rats (p<0.05) compared with the control. However, intervention with both bromelain alone and Cd+bromelain (p<0.05) led to a significant decline in MDA level.

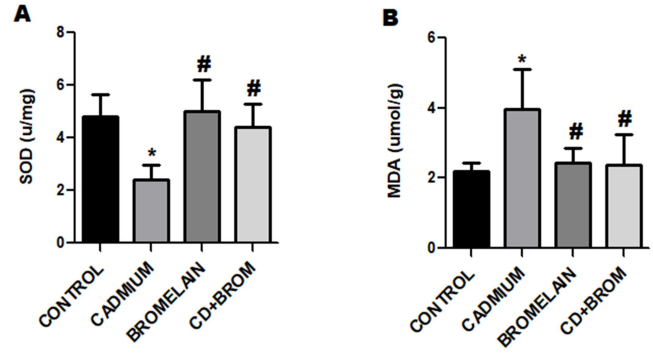

Fig 5: Effects of bromelain on Cd-induced neurotoxicity in rats. A - plasma levels of SOD ; B - plasma levels of MDA. Values are expressed as mean ± SEM (n=5). *P<0.05 vs control, #P<0.05 vs Cd (cadmium).

Effects of Bromelain on PFC Levels of Oxidative Stress Markers in Cd-Induced Neurotoxicity in Rats

SOD: As shown in Figure 6A, the cortical level of SOD was markedly reduced in the Cd (p<0.05) group compared with the control. Rats that received bromelain (p<0.05) and Cd+bromelain (p<0.05) showed an increase in the level of SOD.

MDA: In Figure 6B, the level of MDA in the PFC was markedly increased in the Cd group (p<0.05) compared with control rats. This was reversed both in the bromelain-only and Cd+bromelain (p<0.05) groups when compared with the Cd-only group.

NO: As shown in Figure 6C, the level of nitric oxide was significantly increased in the Cd-only group (p<0.05) compared with the control. In contrast, there was a significant decline in NO in the groups that received bromelain only and Cd+bromelain (p<0.05) compared with the Cd group.

AChE: The cholinergic marker was markedly upregulated in the Cd-exposed rats (p<0.05). In contrast, bromelain (p<0.05) and Cd+bromelain (p<0.05) groups had a decline in the level of AChE, depicting an increase in cholinergic transmission (Fig. 6D).

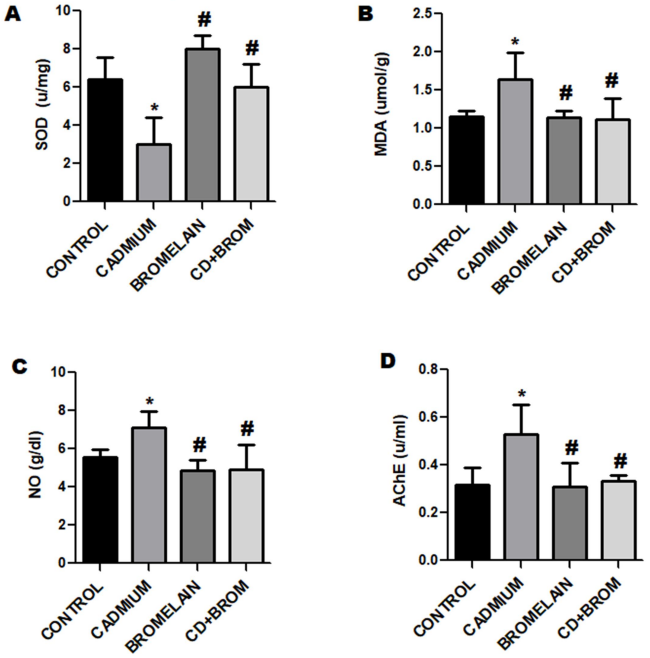

Fig 6: Effects of bromelain on Cd-induced neurotoxicity in rats. A - prefrontal cortical levels of SOD; B - prefrontal cortical levels of MDA; C - prefrontal cortical levels of NO; D - prefrontal cortical levels of AChE. Values are expressed as mean ± SEM (n=5). *P<0.05 vs control, #P<0.05 vs Cd (cadmium)

Effects of bromelain on prefrontal cortical levels of neuro-inflammatory markers in Cd-Induced Neurotoxicity in Rats

IL-6: This was markedly increased in the PFC of Cd-exposed rats (p<0.05) compared with control. Bromelain (p<0.05) and Cd+bromelain showed a marked decline in 1L-6 compared with Cd-exposed rats that received no intervention (Fig 7A).

TNF-α: As shown in Figure 7B, the level of TNF-α was significantly increased in the Cd group (p<0.05) compared with the control. However, treatment with bromelain (p<0.05) and Cd+bromelain led to a marked reduction in its level compared with Cd-exposed rats.

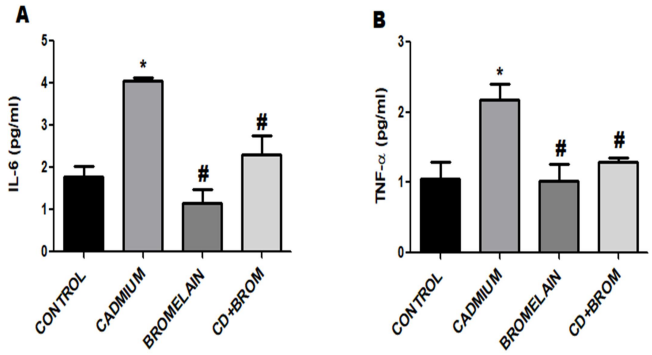

Fig 7: Effects of bromelain on prefrontal cortical levels of IL-6 (A) and TNF-α (B) in Cd-induced neurotoxicity in rats. Values are expressed as mean ± SEM (n=5). *P<0.05 vs control, #P<0.05 vs Cd (cadmium).

DISCUSSION

Cd is neurotoxic and has been implicated in the aetiology of several neurological disorders (Jomova *et al*. 2010). It crosses the blood-brain barrier with relative ease to accumulate in the brain, causing all sorts of behavioural and inflammatory disorders. The use of natural products reported with little to no side effects in the prevention and treatment of diseases has gained significant attention. Several natural compounds from plants have been screened for their neuroprotective effects against metal-induced toxicity. However, bromelain, which has a wide range of scientifically proven therapeutic benefits, has not been investigated in this regard. As such, this study established the ameliorative effects of bromelain on Cd-induced neurobehavioral deficits, cholinergic alteration, neuroinflammation and oxido-nitrosative stress in a rat model.

Several studies have unveiled the neurotoxic effect of Cd and possible mechanisms of its toxicity (Méndez-Armenta and Ríos 2007; Flora *et al*. 2008). Our analyses showed that the residual Cd level in the brains of rats administered Cd only was significantly increased. However, treatment with bromelain significantly reduced Cd levels in the brains of Cd-induced rats. The reduced Cd level could be a result of a metal-ligand relationship between Cd and bromelain, thereby lowering the heavy metal load in the brain. The blood-brain barrier is vital for the functioning of the central nervous system. It forms an organised network of endothelial cells with low permeability. Metal-induced oxidative stress has been reported to interrupt the physiological integrity of the blood-brain barrier (Obermeier *et al*. 2013). This disruption enables toxic substances to gain access to the brain. The accumulation of Cd in the brains of Ca-induced rats observed in this study agrees with previous reports that Cd is associated with leakage of the blood-brain barrier *in vivo* (Oboh *et al*., 2019; Shukla *et al*., 1996).

Behaviours like exploratory drive, cognitive function, anxiety, and depression were evaluated, and the results obtained are consistent with previous reports (Unsal *et al*., 2013; Oboh *et al*., 2019). Rats exposed to Cd showed less exploratory tendency. This is evident in the significant increase in freezing frequency as well as decreases in the number of lines crossed and rearing frequency in rats treated with Cd. On the other hand, rats that were treated with both Cd and bromelain showed significant improvement in their exploratory drive. Moreover, rats that were exposed to Cd exhibited a cognitive deficit, as evidenced by the marked reduction in memory index. This cognitive deficit was improved in rats that received bromelain. Bromelain also improved Cd-induced anxiety-like and depressive-like behaviours. These results showed that bromelain ameliorated neurobehavioural deficits caused by Cd accumulation in the brain. Cd can affect the degree and balance of excitation and inhibition in synaptic neurotransmission, leading to excitotoxicity and synaptic disruption, thus causing behavioural impairments (Mendez-Armenta and Rıos, 2007; Marchetti, 2014). Consistent with our results, studies of occupational exposure to Cd have shown neurobehavioural effects on workers, including a slowing of visuomotor functioning. Increases in complaints about alterations in equilibrium and decreased concentration ability were reported to be dose-dependently associated with urinary Cd levels (Viaene *et al*., 1999).

Appropriate cholinergic neurotransmission provided by ACh is fundamental for the formation of memory (Donovan *et al*., 2014). AChE hydrolyzes acetylcholine, thus diminishing cholinergic neurotransmission. Hence, the assessment of the activity of AChE can be further correlated with cognitive function. In the current work, Cd increased the AChE level, which is consistent with the findings of Carageorgiou *et al*. (2005). It, however, contradicts the decrease in brain AChE activity following Cd exposure reported by El-Tarras *et al*. (2013). Consistent with our results, bromelain reduced hippocampal AChE levels in a mouse model of Alzheimer's disease (Kumar *et al*., 2022). The antioxidant bromelain can modulate the molecular targets involved in brain cholinergic signalling (Eraky *et al*., 2023).

Cd elicits its effects via oxidative stress and inflammation (Oboh *et al*., 2019). Cd enhances the production of ROS and inhibits the activities of antioxidant enzymes (El-kott *et al*., 2020). In tandem with these previous reports, our results showed a significant reduction in plasma and brain levels of SOD. Also, there was a substantial increase in plasma and brain levels of MDA, a marker of lipid peroxidation, and NO, a reactive nitrogen species. Various authors reported that the brains of Cd-intoxicated animals show more significant oxidative stress with a marked depletion of the antioxidants, altered antioxidant enzyme activities, and enhanced lipid peroxidation by different routes of administration and doses applied (Alnahdi and Sharaf, 2019). However, intervention with bromelain caused a significant increase in plasma and brain levels of SOD and a significant decrease in MDA and NO. This shows that bromelain reverses Cd-induced oxidative stress by enhancing the activity of antioxidant enzymes like SOD and lowering the levels of MDA and NO in the brain and plasma. Therefore, it modulates the oxido-nitrosative pathway. Bromelain is a known anti-inflammatory and antioxidant agent (Saptarini *et al*., 2019). Adu *et al*. (2022) showed that treatments of 6-OHDA lesioned rats with bromelain

decreased the plasma concentration of TNF-α and IL-1β. Also, the anti-inflammatory role of bromelain via a reduction in the sciatic levels of TNF-α and IL-1β in a rat model of neuropathic pain has been documented (Bakare and Owoyele, 2021). The present findings also followed a similar trend. There were marked reductions in prefrontal cortical levels of IL-6 and TNF-α in rats that received bromelain intervention following Cd exposure. These results showed that neuro-inflammation caused by Cd accumulation in the brain was reversed by bromelain supplementation.

The observed neuroprotective effects of bromelain can be pinned on the presence of cysteine, an amino acid with known antioxidant properties. Cysteine is an essential precursor in the production of antioxidant glutathione, which protects cells from toxins such as free radicals generated by metal-induced oxidative stress (Piste, 2013). Thus, the cysteine in bromelain could be a factor that acts against oxidative stress.

Chelation therapy has historically been used in attempts to reduce the body burden of toxic metals in patients with highly elevated biological markers (Baum, 1999). The majority of proteins and peptides that function in the uptake, distribution, storage, or detoxification of essential and non-essential metal ions possess one or several metal-binding sites. The Cys-X-X-Cys- and -Cys-Cys- motifs of various proteins are well known for their heavy metal binding properties (Cobbett and Goldsbrough, 2002). It has been documented that sulphydryl-containing compounds like bromelain can chelate metals. The sulphur-containing amino acids methionine and cysteine, as well as the tripeptide glutathione, which also contains cysteine, all contribute to the chelation and excretion of metals from the human body. The presence of a highly reactive cysteine in bromelain could have a plausible chelating effect (Al-Otaibi *et al*., 2015), hence, the observed protection against Cd-induced toxicity.

Conclusion

Bromelain reduced neurobehavioural impairment, cholinergic alteration, and oxido-inflammatory deficits induced by Cd exposure. This study suggests that bromelain may be beneficial in combating Cd-induced neurotoxicity in the brain of rats.

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Conflict of Interest

None declared.

Authors' Contribution

AAB - Conception, design and supervision of the experiment; AKM and AOB - Experimentation and data collection; AAB and TGA - Data analysis and interpretation; AAB, TTA and HA - Drafting of the manuscript; AAB, TTA and BVO - Critical revision of the manuscript.

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